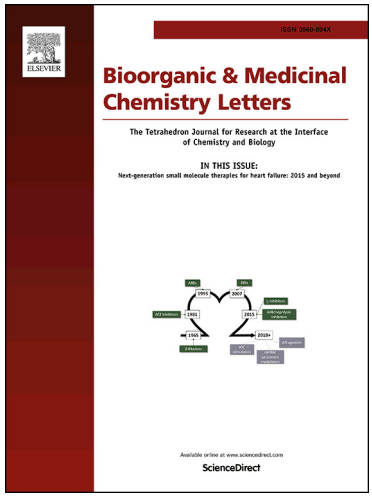

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Design, synthesis and evaluation of curcumin-based fluorescent probes to detect Aβ fibrils

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Key words: Alzheimer's disease, Amyloid β, Curcumin, Inhibitor, Probe

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ABSTRACT: Amyloid β fibrillation is an early event in Alzheimer's disease, so its detection is important to understand its roles in Alzheimer's disease. Curcumin, which has poor water solubility, has been reported to have many pharmacological activities including potent anti-amyloid β fibril activity in Alzheimer's disease. In this study, we found that curcumin analogues with the fluorescence property instead of non-inhibition of amyloid β fibrils. The development of new curcumin analogue, Me-CUR (**9**), as fluorescent switchable probe to detect amyloid β fibrils is described. Me-CUR (**9**) shows excellent fluorescence, especially higher than ThT (**4**), in the presence of amyloid β fibrils. These results suggest that Me-CUR (**9**) can become a useful *in vitro* amyloid fluorescence sensor for diagnosis of Alzheimer's disease.

Amyloid fibrils are insoluble aggregates with fibrillary morphology and a β-sheet rich structure that can cause several diseases known as amyloidosis due to their misfolding[1-3]. Among the different amyloid-like peptides, those called amyloid β (Aβ) are very important because of their prominent role in Alzheimer's Disease (AD)[4,5]. AD is a progressive neurodegenerative disorder characterized by cognitive decline, irreversible memory loss, disorientation and language impairment[6,7]. One of the pathological hallmarks is the aggregation of Aβ peptide and its accumulation in the human brain has an important role in the etiology of AD[8]. Therefore, Aβ fibrils could be a biomarker for therapy or diagnosis for AD. Although there are several isoforms of Aβ species ranging in length from 39 to 43 residues, Aβ40 and Aβ42 are the most abundant (9:1 = Aβ40:Aβ42) and Aβ42 is more aggregation-prone[9]. Therefore, inhibiting or disrupting the Aβ42 aggregation process represents a promising therapeutic strategy for the prevention and treatment of AD.

Curcumin (CUR (**1**)), is a yellow pigment exacted from *Curcuma longa* (turmeric), which is commonly used as a food colorant, spice, and cosmetic, as well as a traditional medicine in Asian countries[10,11]. In the last few decades, CUR (**1**) has become a research focus due to its numerous beneficial biological and pharmacological activities such as antioxidant, anti-tumor, anti-inflammatory and other desirable medicinal benefits[12-17]. CUR (**1**) is one of the PAINS (pan assay interference compounds) and IMPS (invalid metabolic compounds), and more than 1,000 curcuminoids have been developed to improve their activities to date[18-20]. Especially, the effect of anti-Aβ fibrils as highly beneficial in the treatment of AD has been reported by the Ono group[21]. CUR (**1**) has a characteristic structure in which two phenolic groups are linked by an olefin chain containing a β-diketone moiety and the high crystallinity reduces the solubility to solvents markedly[22,23]. Since the insolubility of CUR (**1**) in water may restrict its pharmacodynamics, we have successfully synthesized a water-soluble CUR analogue (Ws-CUR (**2**)), with potent inhibitory activity against Aβ fibrils. Ws-CUR (**2**) shows 31 μM of water-solubility and has intensive inhibitory activity of Aβ fibrils[24].

To date, the most widely applied Aβ imaging probes have used positron emission tomography (PET) and single photon emission computed tomography (SPECT) using Congo Red (CR, (**3**)) and Thioflavin T (ThT (**4**))[25,26]. CUR (**1**) has also been developed as a PET imaging dye or Near-infrared (NIR) dye; however, these imaging modalities

are hindered by their intrinsic high cost and time-consuming nature and result in patient exposure to radiation[27-29]. Therefore, labeling technology has been increasingly popular for imaging of Aβ fibrils as a potential alternative to other techniques[30,31]. Among these molecular probes, ThT (**4**) is the most widely used dye for monitoring amyloid fibrils both *in vitro* and *in vivo*[32]. Although the ThT (**4**) binding model is largely unknown, the increasing emission intensity of ThT (**4**) upon binding to amyloid fibrils is due to the inhibition of the rotation around a single C-C bond in ThT (**4**)[33]. Therefore, there is disadvantage in the point of low material selectivity. Moreover, CRANAD-2 (**5**) for NIR probe and Shiga-Y5 (**6**), ^{19}F-MR diagnostic imaging probe, have the CUR-structure, so these results suggest that it is possible they can detect Aβ fibrils as a fluorescent molecular rotor to CUR (**1**)[34], which has five single C-C bonds. The property of CUR (**1**) is expected high selectivity and potent detection capability. In addition, most CUR (**1**) and its analogues were symmetry structures in the previous work by many researchers[18-20]. Development of Ws-CUR (**2**) by our group indicated that asymmetry CUR structures exploit novel ability. Although a variety of organic dyes were reported, a few compounds have been applied as biomarker of Aβ fibrils. It is anticipated that CUR (**1**) and its analogues rarely show aggregation-induced emission (AIE) and can be reversibly showed AIE efficacy depending on the aggregation state of Aβ fibrils. Herein, we report the design, synthesis and evaluation of CUR analogues as new fluorescence probes (Figure **1**).

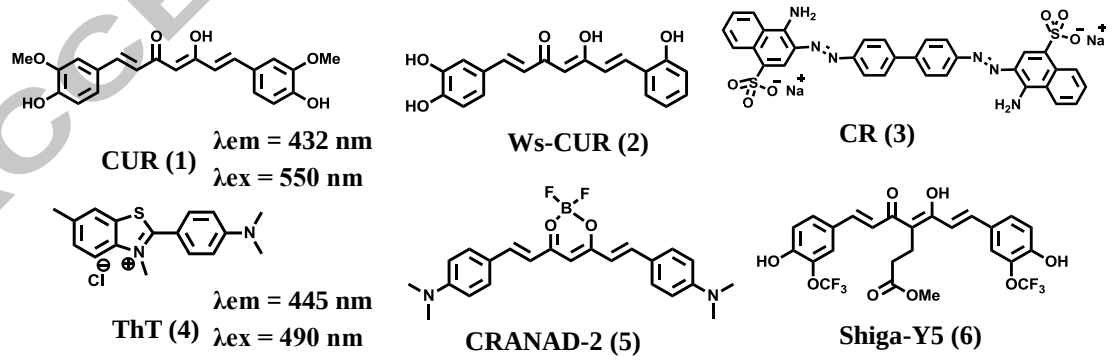


Figure 1. Structure of CUR (1), Ws-CUR (2), CR (3), ThT (4), CRANAD-2 (5) and Shiga-Y5 (6)

In our previous work, we performed a structure activity relationship study (SAR) to develop an Aβ aggregation inhibitor. As depicted in Figure 2, inhibitory activity of

Me-CUR (**7**) against Aβ fibrils decreased markedly compared with those of Ws-CUR (**2**). In addition, ThT-induced fluorescence intensity of Me-CUR (**7**) with Aβ fibrils was more intense than that in the absence of CUR derivatives as a positive control (Figure 2).

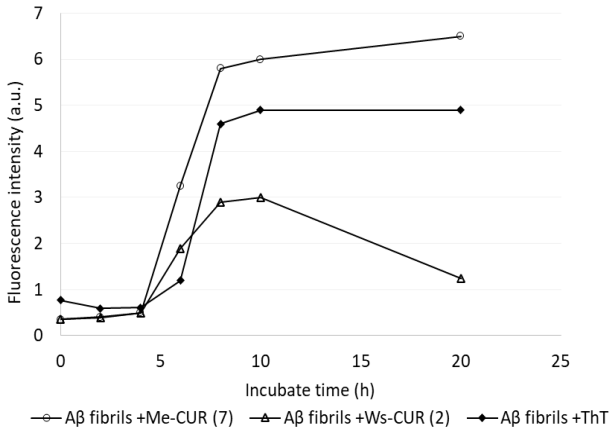
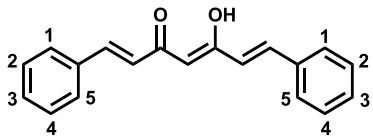


Figure 2. Time-dependent fibril formation of Aβ42 was monitored by ThT (4), Me-CUR (7) and Ws-CUR (2) (dye 5 μM, Aβ42 25 μM).

As for this phenomenon, we assume that Me-CUR (**7**) has no inhibition activity for Aβ fibrils and may not induce their collapse. To investigate this phenomenon, we designed CUR analogues, which converted the hydroxy group into methoxy group of phenyl rings to develop more effective Me-CURs as fluorescence probes for Aβ fibrils. Moreover, Ws-CUR (**2**), which has an ortho hydroxy group, showed potent inhibition activity even though Ws-CUR (**2**) has one less hydroxy group than CUR (**1**). Therefore, we designed CUR analogues with two or three methoxy groups on their phenolic rings. Especially, the necessity for the methoxy group in the ortho-position of Me-CURs was verified to detect Aβ fibrils. Me-CURs (**7**) - (**18**) with four methoxy groups on their phenolic rings and a similar structure to CUR, were also investigated (Table 1). To prepare the designed compounds (**7**) - (**18**), aldol condensation of an acetylacetone-boron complex and benzaldehyde derivatives in the presence of piperidine or *n*-buthylamine is a key step (See supporting information SI-Scheme 1). Symmetrical derivatives were synthesized directly and asymmetric derivatives were synthesized twice by repeating the operation.



Ar1 **Ar2**

Table 1. Molecular design of Me-CURs.

OMe[a]	Compound	Ar1	Ar2
2	**7**	2	2
	8	3	3
3	**9**	3,4	2
	10	3,4	4
4	**11**	2,3	2,3
	12	2,3	2,5
	13	2,5	2,5
	14	3,4	2,3
	15	3,4	2,5
	16	3,4	3,4
	17	3,4,5	2
6	**18**	3,4,5	3,4,5

a: The number of methoxy groups.

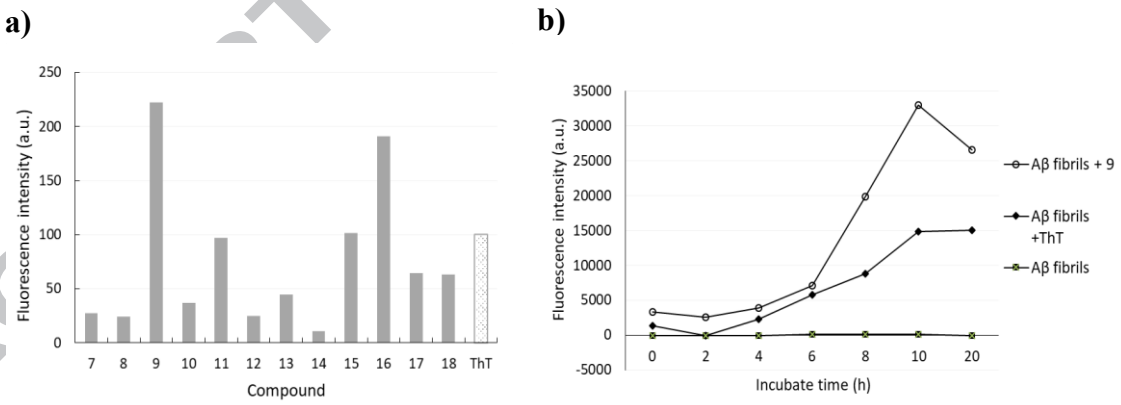


Figure 3. (a) Fluorescence quantities of Me-CURs (7) - (18) for Aβ fibrils (Incubate time, 10h, ThT fluorescence, 100) (b) Time-dependent fibril formation of Aβ42 was monitored by ThT compared with 9 (dye 5 μM, Aβ42 25 μM).

Evaluation of synthesized molecules, Me-CURs (**7**) - (**18**), as fluorescence probes for Aβfibrils was performed by an incubation assay with aggregated Aβ42 peptide. ThT (**4**), the control probe, is a benzothiazole dye that exhibits enhanced fluorescence upon

binding to amyloid fibrils. As shown in Figure 3a, fluorescence intensities of Me-CURs (**9**), (**15**) and (**16**) with the Aβ fibrils were stronger than those of ThT (**4**). In the spectra of the Aβ42-ThT assay system in Figure 3b, **9** showed constant weak autofluorescence. In contrast, **9** behaved as selective light-up fluorescent probe upon binding to the Aβ fibrils, as fluorescence intensity was increasing. In other words, **9** can be selectively and reversibly turned on and off depending on the aggregation state of Aβ fibrils and act as an Aβ fibrils-responding On-Off switchable fluorescent probe (Figure 3).

To investigate the spacer unit of the CUR framework, ‘‘Downsizing’’ derivatives of Me-CUR (**9**), which have a short olefin linker and reduced number of ketones, were designed. Basically, the C7-diketone olefin linker of CUR framework was replaced with C5-diketone, C5-monoketone and C3-monoketone olefin linkers to afford downsized analogues (**19**), (**20**) and (**21**). These downsized analogues (**19**), (**20**) and (**21**) were synthesized as shown in SI-Scheme 2 (See supporting information SI-Scheme 2). A List-Barbas aldol reaction was employed to give C5-diketone derivatives (**19**) and C5-monoketone derivatives (**20**) as a key step. The downsizing analogue (**20**) was prepared by a base-catalyzed aldol condensation reaction starting from *o*-anis aldehyde. C3-monoketone derivatives (**21**) were synthesized by coupling of *o*-anis aldehyde and *o*-methoxy acetophenone in the presence of sodium methoxide. As shown in Figure 4, these short-linker Me-CURs (**19**), (**20**) and (**21**) showed weaker or undetectable fluorescence. The results suggested that the methoxy groups at the 3,4-positions of

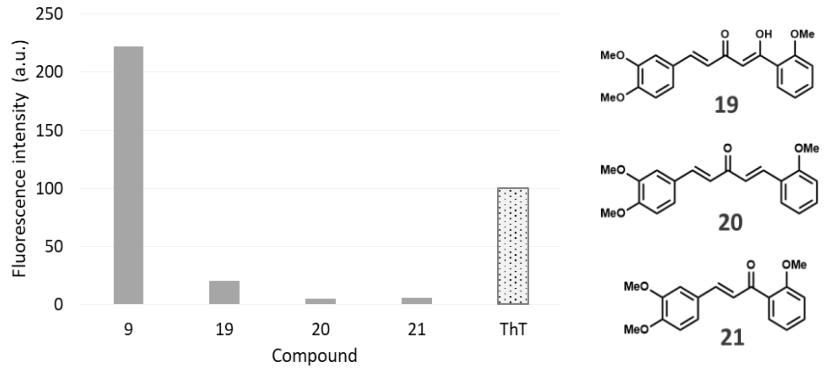


Figure 4. “Downsizing” analogues of 9; C5-diketone type derivative (19); C5-monoketone type derivative (20); C3-monoketone type derivative (21)

phenolic rings and olefin chain having both 7 carbon atoms and the β-diketone motif as CUR are needed to recognize the Aβ fibrils (Figure 4).

We also investigated the photophysical behavior of **9**, **15** and **16**. There was a significant association between the mechanism to stain Aβ fibrils and the molecular planarity as ThT (**4**) behaves like a "molecular rotor". In detail, a low energy barrier allows the benzenamine and benzothiazole rings of ThT (**4**) to rotate freely about their shared C-C bond in solution. Amyloid fibrils are likely to present a ThT-binding site that sterically "locks" the bund dye, thus leading to enhancement of ThT fluorescence. Me-CURs also have the methoxy groups on phenyl rings, which are linked to the olefin chain via two single bonds. Thus, they feature high rotational flexibility and possesses two different twisted intramolecular change transfer (TICT) channels within the whole molecular structure. These intramolecular rotations lead to the non radiative deactivation of the fluorescent excited state, which could be another cause for the weak auto fluorescence of Me-CURs. To test this assumption, we investigated the effect of solvent viscosity on Me-CUR emission. The intramolecular rotation process is reported to be influenced by the viscosity of the medium; in a high viscosity medium, the intramolecular rotation was slowed down and hence enhanced the emission of Me-CURs (**9**), (**15**) and (**16**). Thus, we examined the viscosity effect on the emission behavior of Me-CURs (**9**), (**15**) and (**16**) which detected Aβ fibrils more sensitively than ThT (**4**) in glycerol. As shown in Figure. 5b, the fluorescence intensity of Me-CURs (**9**), (**15**) and (**16**) were dramatically enhanced with a solvent viscosity which is typically observed for molecular rotors like ThT (**4**). These results support the fact that Me-CURs act as the molecular rotor and could turn on/off their emission when free rotation of phenyl rings to the olefin linker is restricted (Figure 5b). Based on this hypothesis, we calculated the molecular planarity of Me-CURs (**9**), (**15**) and (**16**) when the two carbon-carbon bonds sharing both sites of aromatic rings and the olefin linker of Me-CURs (**9**), (**15**) and (**16**) defined as their rotor (switch). As we expected, Me-CUR (**9**) had a high molecular planarity (Φ= 8.8) compared with **15** (Φ= 79.3) and **16** (Φ= 81.7) (Figure 5a). Thus, the high molecular planarity of Me-CUR (**9**) is an important factor in the robust intense fluorescence when interacting with Aβ fibrils. Moreover, Me-CUR (**9**) showed sufficient stokes shift to use as a fluorescence probe (Figure 5c).

The photoproperties of Me-CURs (**9**), (**15**) and (**16**) are shown in Figure 5c. To obtain more emission properties of Me-CUR (**9**), a concentration titration test was done. The fluorescence emission maxima of Me-CUR (**9**) shifted from 520 nm to 496 nm in the presence of Aβ fibrils from 0.5 μM to 25 μM as shown in Figure 5d. The concentration dependent-fluorescence spectral changes of Me-CUR (**9**) showed that the fluorescence intensity gradually increased along with increasing concentrations of Aβ fibrils, accompanied by a slight blue shift in λ_{max}, while it did not change markedly in the solution without Aβ fibrils (Figure 5). There is sigmoidal curve between fluorescence intensity shift (nm) and concentration of Aβ fibrils (μM) and consequently, Aβ concentration at a range of 5-10 μM prompts to a blue shift in λ_{ma} (See supporting information SI-Figure 2). As further investigation, the detection limit between Aβ fibrils and Me-CUR (**9**) was estimated on fluorescence intensity. To detect 25 μM Aβ fibrils, Me-CUR (**9**) from 0.2 μM to 500 μM were attempted in standard condition. More than 10 μM Me-CUR (**9**) gave saturated data (>60000 a.u.) and 0.2 μM Me-CUR (**9**) shows no detection with 25 μM Aβ fibrils. On the other hand, we confirmed that less than 6 nM Aβ fibrils was hardly performed fluorescent detection using 10 μM Me-CUR (**9**) and, therefore, it needs to exist more than 60 nM Aβ fibrils to exhibit the ability of Me-CUR (**9**) (See supporting information SI-Figure 3).

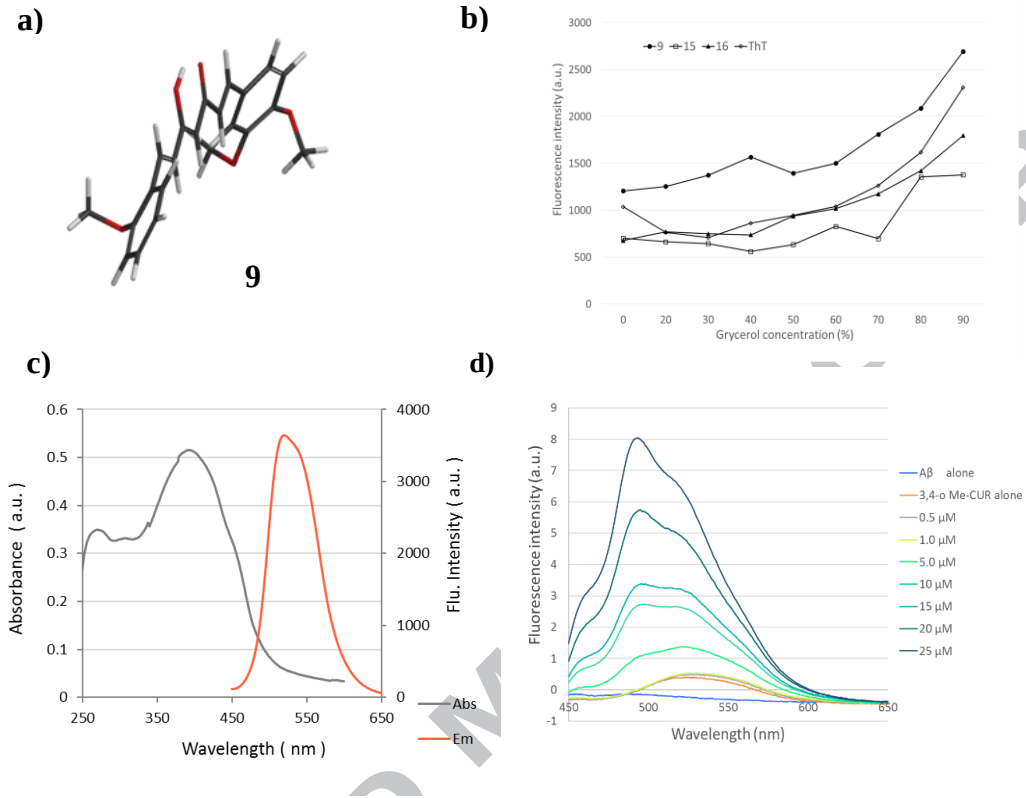


Figure 5. (a) The most stable conformation of Me-CUR (9). (b) Viscosity-dependent emission behavior of Me-CUR (9), (15) and (16) were observed compared with ThT. (c) The absorption and emission spectra of Me-CUR (9). (d) Concentration dependent-fluorescence titration. Aβ42 0.5 - 25 μM, Me-CUR (9), 5 μM.

To observe the morphology of Aβ fibrils, the effect on amyloid formation was also investigated by TEM (Figure. 6a-f). Aβ42 formed amyloid fibrils of 30-120 nm diameter and 300 nm-2 μm long owing to aggregation properties (Figure 6a). The addition of ThT (**4**) to Aβ fibrils maintained the fibril formation as a positive control (Figure 6b). The addition of Me-CUR (**9**) kept the formation of fibrils from transforming and fibril formations of 300-500 nm in length were still observed, which corresponded to the fluorescence intensity (Figure 6c). In addition, the other Me-CUR analogues, which have short olefin linkers, C5-diketone type derivative (**19**), C5-monoketone type derivative (**20**), C3-monoketone type derivative (**21**) were also analyzed. The results indicated that the addition of the C5-diketone type derivative (**19**) led to some shorter fibril formation (~200 nm) (Figure 6d), and the addition of the C5-monoketone type derivative (**20**) and C3-monoketone (**21**) derivative changed the morphology of Aβ fibrils (Figure 6e,f). To visualize the interaction of ThT (**4**) or Me-CURs (**9**), (**19**)-(**21**)

with Aβ fibrils, we observed fibril formation by fluorescence microscopy (Figure 6g-l). Aβ fibrils showed no fluorescence with an excitation filter (B) of 460-495 nm as a negative control (Figure 6g). The fluorescence of ThT (**4**) was clearly evident on the amyloid aggregate and it showed as a green light (Figure 6h). We found that our promising probe, Me-CUR (**9**), stained the fibrous aggregates of Aβ fibrils with a yellow light (Figure 6i). Predictably, the C5-diketone type derivative (**19**) undetected the aggregates, and only confirmed their autofluorescence. Neither the C5-monoketone type derivative (**20**) nor the C3-monoketone type derivative (**21**) also showed any fluorescent activity to bind to the aggregates (Figure 6j-l).

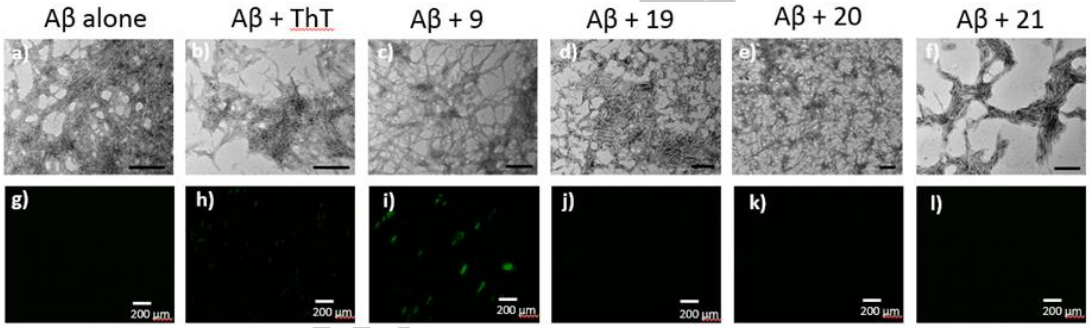


Figure 6. TEM images of the Aβ fibrils (a-e). (a) Aβ fibrils. (b) the addition of ThT. (c) the addition of 9. (d) the addition of 19. (e) the addition of 20. (f) the addition of 21. Fluorescence microscopy analyses of the Aβ fibrils (g-l). (g) Aβ fibrils. (h) stained with ThT. (i) stained with 9. (j) stained with 19. (k) stained with 20. (l)stained with 21.

To predict insight into the possible interaction mechanism for Aβ fibrils binding, we performed molecular docking analysis. The binding mode of Me-CUR (**9**) or ThT (**4**) was achieved with Aβ fibrils using GOLD software on a 12-fold Aβ1-42 fibril structure (PDB ID: 2MXU). As shown in Figure 7, Me-CUR (**9**) and ThT (**4**) located in the inner site of Aβ fibrils (Figure 7a,b). The binding mode between Me-CUR (**9**) or ThT (**4**) and the Aβ fibrils was mainly supported by the hydrophobic interaction. In detail, several significant binding interactions were observed within the docked complex, as depicted in Figure 7c,d. Our docking studies *in silico* further supported the fact that the localization site of Me-CUR (**9**) and ThT (**4**) are similar and are sustained by hydrophobic interaction and hydrogen bonding networks (Figure 7).

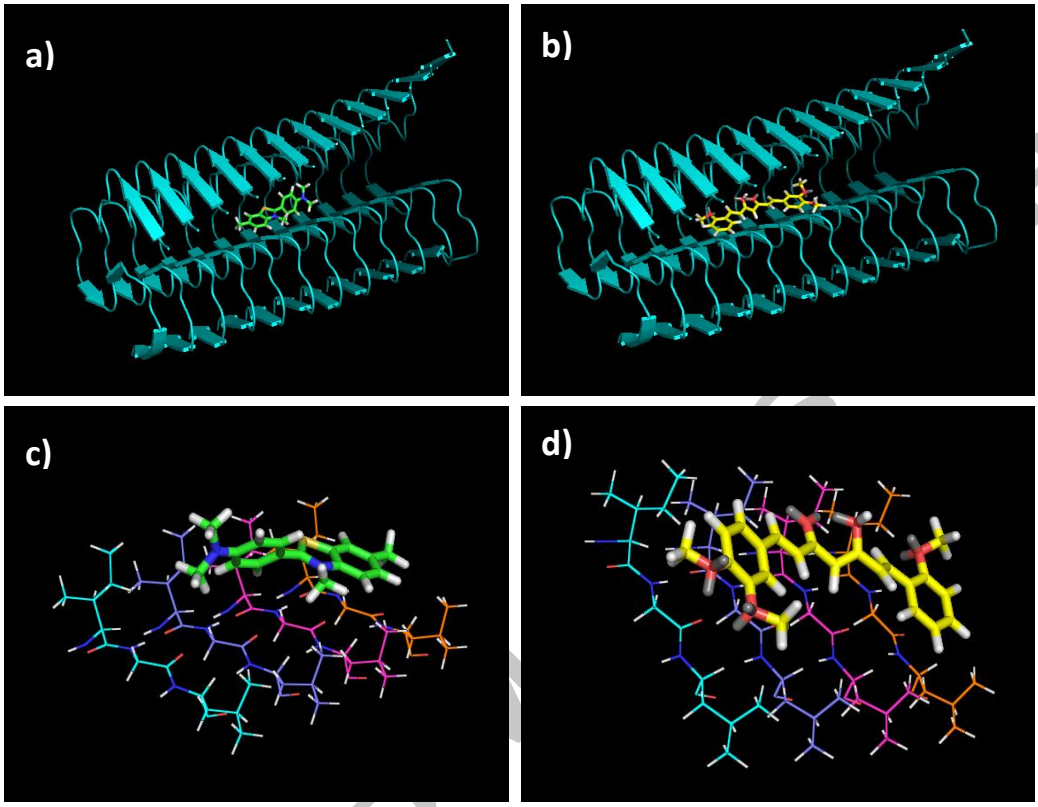


Figure 7. (a) Binding pose of ThT (4) in the Aβ fibrils; (b) Binding pose of 9 in the Aβ fibrils; (c) Detailed view of the docked ThT structure and the corresponding interacting amino-acid moieties within the binding site of Aβ fibrils; (d) Detailed view of the docked 9 structure and the corresponding interacting amino-acid moieties within the binding site of Aβ fibrils.

In summary, a new series of novel curcumin analogues, an Me-CUR library containing short linker moiety or various substituents, was successfully synthesized and evaluated as new imaging agents for Aβ fibrils. The Me-CURs, (**9**), (**15**), (**16**), which have a C7-diketone moiety and phenolic rings containing methoxy groups at 3,4-positions, showed higher fluorescence than ThT (**4**) as the most extensive amyloid sensor. Detailed spectroscopic studies indicated that Me-CUR (**9**) has high molecular planarity and thus it could strongly bind with the Aβ fibrils resulting in a large increase in its emission intensity with a large spectral shift. Me-CUR (**9**) is a fluorescent switchable probe to detect amyloid β fibrils with high sensitivity. At this time, Me-CUR (**9**) can be employed as the detecting agent of Aβ fibrils in vitro experiment instead of

ThT (**4**). These results suggest that Me-CUR (**9**) can become a useful fluorescence probe for diagnosis of AD. Our research using cell lines and a serum is in progress.

Supporting information

Additional experimental procedures, and analytical data for synthetic compounds. This material is available free of charge via the Internet at xxxxxxx.

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Design, synthesis and evaluation of curcumin-based fluorescent probes to detect Aβ fibrils

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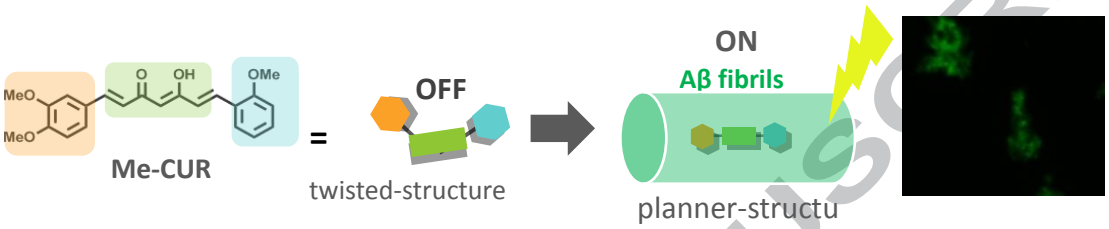

Design, synthesis and evaluation of curcumin-based fluorescent probes to detect Aβ fibrils

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Highlights:

Me-CUR is a fluorescent switchable probe to detect amyloid β fibrils with high sensitivity

Me-CUR can be employed as the detecting agent of Aβ fibrils in vitro experiment instead of ThT

Me-CUR has high molecular planarity and thus it could strongly bind with the Aβ fibrils